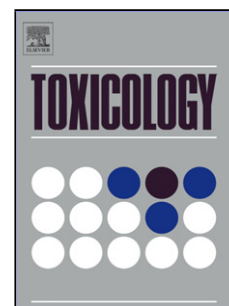


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Aluminum exposure at human dietary levels promotes vascular dysfunction and increases blood pressure in rats: a concerted action of NAD(P)H oxidase and COX-2

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Abbreviations: MRA, mesenteric resistance arteries; ACh, acetylcholine; Phe, phenylephrine; SOD, superoxide dismutase; TXA-2 R, thromboxane A2 receptor; nitric oxide synthase, NOS; L-NAME, N ω -nitro-L-arginine methyl ester, inhibitor of NOS; TEA, tetraethylammonium, potassium channels blocker; NS 398, selective COX-2 inhibitor; SNP, sodium nitroprusside.

Highlights

- **Al at human dietary levels reaches a threshold to induce cardiovascular dysfunction.**
- **Al exposure at low doses promotes vascular dysfunction and increases blood pressure.**
- **Al induces endothelial dysfunction and impairs endothelial NO bioavailability.**
- **This study raises the concern regarding safe values for human exposure to Al.**

Abstract

Aluminum (Al) is a non-essential metal and a significant environmental contaminant and is associated with a number of human diseases including cardiovascular disease. We investigated the effects of Al exposure at doses similar to human dietary levels on the cardiovascular system over a 60 day period. *Wistar* male rats were divided into two major groups and received orally: 1) Low aluminum level - rats were subdivided and treated for 60 days as follows: a) Untreated - ultrapure water; b) AlCl_3 at a dose of 8.3 mg/kg bw for 60 days, representing human Al exposure by diet; and 2) High aluminum level - rats were subdivided and treated for 42 days as follows: C) Untreated – ultrapure water; d) AlCl_3 at 100 mg/kg bw for 42 days, representing a high level of human exposure to Al. Effects on systolic blood pressure (SBP) and vascular function of aortic and mesenteric resistance arteries (MRA) were studied. Endothelium and smooth muscle integrity were evaluated by concentration-response curves to acetylcholine (ACh) and sodium nitroprusside. Vasoconstrictor responses to phenylephrine (Phe) in the presence and absence of endothelium and in the presence of the NOS inhibitor L-NAME, the potassium channels blocker TEA, the NAD(P)H oxidase inhibitor apocynin, superoxide dismutase (SOD), the non-selective COX inhibitor indomethacin and the selective COX-2 inhibitor NS 398 were analyzed. Vascular reactive oxygen species (ROS), lipid peroxidation and total antioxidant capacity, were measured. The mRNA expressions of eNOS, NAD(P)H oxidase 1 and 2, SOD1, COX-2 and thromboxane A2 receptor (TXA-2 R) were also investigated. Al exposure at human dietary levels impaired the cardiovascular system and these effects were almost the same as Al exposure at much higher levels. Al increased SBP, decreased ACh-induced relaxation, increased response to Phe, decreased endothelial modulation of vasoconstrictor responses, the bioavailability of nitric oxide (NO), the involvement of potassium channels on vascular responses, as well as increased ROS production from NAD(P)H oxidase and contractile prostanoids mainly from COX-2 in both aorta and mesenteric arteries. Al exposure increased vascular ROS production and lipid peroxidation as well as

altered the antioxidant status in aorta and MRA. Al decreased vascular eNOS and SOD1 mRNA levels and increased the NAD(P)H oxidase 1, COX-2 and TXA-2 R mRNA levels. Our results point to an excess of ROS mainly from NAD(P)H oxidase after Al exposure and the increased vascular prostanoids from COX-2 acting in concert to decrease NO bioavailability, thus inducing vascular dysfunction and increasing blood pressure. Therefore, 60-day chronic exposure to Al, which reflects common human dietary Al intake, appears to pose a risk for the cardiovascular system.

Keywords: metal; cardiovascular risk; vascular impairment; oxidative stress.

1. Introduction

Aluminum (Al) is the most common metal in the Earth's crust and its versatile properties as several Al compounds bring us in direct contact with this non-essential metal (Exley 2012). Human exposure to Al through myriad ways is inevitable (Exley 2013). In 2007, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) followed in 2008 by the European Food Safety Authority (EFSA), have adjusted the Provisional Tolerable Weekly Intake (PTWI) of Al for humans to 1 mg Al/Kg body weight (b.w.) (EFSA 2008; WHO 2007). However, it is known that the PTWI of Al may be exceeded by humans (Fekete et al. 2013; Gonzalez-Weller et al. 2010; Yang et al. 2014). Despite its ubiquity Al has no known biological function, it is not beneficial and it is only toxic (Exley 2009). Therefore, the burgeoning increase in human exposure to Al has been concomitant with an increased development of Al-related adverse effects (Exley 2012; Klein et al. 2014). The accumulation of Al compounds in the human body has been associated with several conditions such as neurological disorders (Crépeaux et al. 2017; Mirza et al. 2016), macrophagic myofascitis (Gherardi et al. 2016), microcytic anemia (Barata et al. 1996) and osteopenia (Li et al. 2011).

Previous studies have paid attention to the relationship between Al exposure and cardiovascular risk (Korchazhkina et al. 1999; Lind et al. 2012; Subrahmanyam et al. 2016). In the last years, several cohort studies have supported a possible role for Al in cardiovascular disease (Costello et al. 2014; Neophytou et al. 2016; Subrahmanyam et al. 2016). For example, research has shown that exposure to airborne particles of Al with an aerodynamic diameter $<2.5 \mu\text{m}$ are a strong predictor of ischemic heart disease risk in Al industry workers (Costello et al. 2014; Neophytou et al. 2016). Al appears to have high affinity to human endothelial cells showing an ability to be accumulated in several human arteries (Bhattacharjee et al. 2013; Minami et al. 2001). Moreover, the circulating presence of Al seems to be a contributor for arterial stiffness

in healthy individuals (Subrahmanyam et al. 2016) and to atherosclerosis plaques formation in the elderly (Lind et al. 2012).

These epidemiological studies are supported by a number of experimental studies. Animals exposed to up to 256 mg Al/kg bw over 120 days showed an increase of blood pressure and impairments in erythrocyte membranes (Zhang et al. 2015). Moreover, a single intraperitoneal injection of Al at 0.5 mg/kg promoted up-regulation of renin expression in rats (Ezomo et al. 2009). At the vascular bed, our group has recently reported that an acute exposure to Al was sufficient to promote vascular dysfunction with a reduction of vascular reactivity (Schmidt et al. 2016). Data thus far in this area have been obtained in studies where the exposure to Al might be considered as unrealistically high. Therefore we have looked to address this discrepancy by studying cardiovascular function in rats exposed to both a high level of Al and also one that better mimics human exposure to Al in the diet.

2. Materials and methods

2.1 Animals

Three-month-old male *Wistar* rats (360 ± 11.2 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12h light-dark), giving free access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguai, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups, according to Martinez et al. (2017) and treated orally: 1) Low aluminum level - rats were subdivided (N=10/each) and treated for 60 days as follows: a) Untreated – received ultrapure water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA) by drinking water; b) AlCl_3 at 8.3 mg/kg bw per day, a dose based on human dietary levels translated to an animal dose according to the body surface area normalization method (Reagan-Shaw et al. 2008); and 2) High aluminum level - rats were subdivided (N=10/each) and treated for 42 days as follows: a) Untreated – received ultrapure water through oral gavage; b) AlCl_3 at 100 mg/kg bw per day through oral gavage once a day, representing a high level of human exposure to Al (Prakash and Kumar 2009).

$\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water. The concentration of each stock solution was 0.034 M and 0.331 M, respectively from 8.3 and 100

mg/kg bw. Salts and reagents were of analytical grade obtained from Sigma-Aldrich and Merck (Darmstadt, Germany).

2.2 Systolic Blood Pressure

Indirect systolic blood pressure (SBP) was measured weekly, before the start of the treatment and during all the treatment period, using non-invasive tail-cuff plethysmography according to Wiggers et al. (2008) (AD Instruments Pty Ltd, Bella Vista, NSW, Australia).

2.3 Vascular reactivity experiments

Rat body weight, feed and water or AI intakes were measured weekly. At the end of the treatments, animals were anaesthetized with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *ip*) and euthanized. Thereafter, the thoracic aorta and the third-order MRA were carefully dissected out and cleaned of fat and connective tissues. For vascular reactivity experiments, in the day of the euthanasia, the arteries were divided into cylindrical segments of 2 mm in length and placed into Krebs-Henseleit solution (in mM: NaCl 118; KCl 4.7; NaHCO₃ 23; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O₂ and 5% CO₂ (pH 7.4). The remaining aorta and mesenteric resistance arteries (MRA) were kept at –80°C for further biochemical/biological assays.

Segments of aorta were mounted in an isolated tissue chamber and maintained at a resting tension of 1.5 g at 37°C. Isometric tension was recorded using an isometric force transducer (TSD125BX8, Biopac Systems, Inc, Santa Barbara, CA, USA) connected to an acquisition system (MP150WSW-SYS, Biopac Systems). MRA segments were mounted in a small-vessel dual chamber myograph (Multi Wire Myograph System, DMT620, ADInstruments, Australia) for measurement of isometric tension according to Wiggers et al. (2008). Segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-to-wall tension ratio of the segments by setting their internal circumference, *L_o*, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg.

After a 45-min equilibration period, aortic and MRA segments were respectively exposed twice to 75 and 120 mM KCl, first to check their functional integrity and again to assess the maximal tension developed. Afterwards, endothelial integrity was tested with acetylcholine (ACh, 10 µM) in segments that were previously contracted with phenylephrine (Phe) at a concentration that produced close to 50% of the contraction induced by KCl. After 60 min of washout, a single concentration response curve to Phe (0.01 nM – 300 µM) was performed.

To evaluate the role of the endothelium in the vasoconstrictor responses to Phe, this vascular component was mechanically removed, and its absence was confirmed by the inability of ACh to induce relaxation greater than 10% of the previous contraction due to Phe. To evaluate the participation of nitric oxide (NO), potassium channels, ROS, prostanoids or AT1 receptors on Phe responses, the effects of the nonspecific nitric oxide synthase (NOS) inhibitor N ω -nitro-L-arginine methyl ester (L-NAME 100 μ M), the potassium channels blocker tetraethylammonium (TEA, 2 mM), the NADPH oxidase inhibitor apocynin (0.3 μ M), the superoxide dismutase (SOD 150 U/ml), the nonselective COX inhibitor indomethacin (1 μ M), the selective COX-2 inhibitor NS 398 (1 μ M) were investigated by their addition 30 min before Phe in vessels with intact endothelium.

To evaluate the endothelial dependent and independent relaxations, concentration-response curves with ACh (0.01 nM – 300 μ M) and sodium nitroprusside (SNP, 0.01 nM – 300 μ M), respectively, were performed in segments previously contracted with Phe.

2.4 Aluminum content in blood and liver

The Al content of blood and liver was determined using an established method (House et al. 2012). Briefly, approximately 0.5g of liver was dried to a constant weight at 37°C. Dried and weighed tissues and 1 mL of blood, were digested in a 1:1 mixture of 15.8M HNO₃ and 30% w/v H₂O₂ in a microwave oven (MARS Xpress CEM Microwave Technology Ltd) and the Al content of digests measured by TH GFAAS (Transversley Heated Graphite Furnace Atomic Absorption Spectrometry). Results were expressed as μ g Al/L of blood or μ g Al/g dry tissue. Each determination was the arithmetic mean of a triplicate analysis.

2.5 Reactive oxygen species levels

Biochemical studies of oxidative stress biomarkers were performed in aorta and MRA. For that, vessels were homogenized in 50 mM Tris HCl, pH 7.4, centrifuged at 2400g for 10 min at 4°C and the resulting supernatant fraction was used for the measurements.

Levels of reactive species were determined by the spectrofluorometric method described by Loetchutinat et al. (2005). This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The supernatant fraction of the sample was diluted (1:10) in 50 mM Tris HCl (pH 7.4) and 2', 7'-dichlorofluorescein diacetate (DCHF-DA; 1mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity

emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence units.

2.6 Lipid peroxidation

Lipid peroxidation was measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. (1979), with modifications (Martinez et al. 2017). An aliquot of each tissue was incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4), and sodium dodecyl sulphate 0.8% (SDS) at 100°C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as nanomoles of MDA per mg of protein.

2.7 Ferric Reducing/Antioxidant Power (FRAP) Assay

The total antioxidant capacity was measured by FRAP assay (Benzie and Strain 1996), with modifications (Martinez et al. 2017). This method is based on the ability of samples to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-pyridil)-s-triazine (TPTZ) the chelate complex Fe^{2+} -TPTZ. Briefly, 10 μL of the supernatant fraction of each tissue was added to 1 mL freshly prepared and pre-warmed (37°C) FRAP reagent (300mM acetate buffer (pH = 3.6), 10mM TPTZ in 40mM HCl, and 20mM FeCl_3 in the ratio of 10:1:1) in a test tube and incubated at 37°C for 10min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50-1000 μM – water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

2.8 In situ detection of vascular $\text{O}_2^{\cdot-}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* superoxide radical anion ($\text{O}_2^{\cdot-}$) production in both aortic and mesenteric segments, as previously described (Briones et al. 2009). Dihydroethidine freely permeates cells and is oxidized in the presence of $\text{O}_2^{\cdot-}$ to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum at 600–700 nm. Frozen tissue segments were cut into 10 μm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mM/L: 130 NaCl, 5.6 KCl, 2 CaCl_2 , 0.24 MgCl_2 , 8.3 HEPES, 11 glucose, pH=7.4). Fresh buffer containing DHE (2×10^{-6} M/L) was applied topically into each tissue section, cover-slipped, incubated for 30 min in a light protected humidified chamber at 37°C and then viewed with a fluorescent microscope

(Zeiss Axioskop 2 microscope - Zeiss, Jena, Germany- Leica TCS SP2 equipped with a krypton/argon laser, x20 objective, zoom 4x), using the same imaging settings in control and arteries from AI-treated rats. Fluorescence was detected with a 568 nm long-pass filter. For quantification, five rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated using NIH Image J software version 1.46r (<http://rsbweb.nih.gov/ij/>), using the same imaging settings in each case.

2.9 Lucigenin Chemiluminescence Assay

A lucigenin-enhanced chemiluminescence assay was used to determine NAD(P)H (NOX) activity. Aortas were homogenized in lysis buffer (50 mM KH₂PO₄, 1 mM EGTA, and 150 mM sucrose, pH 7.4). The reaction was started by the addition of NADPH (0.1 mM) to the suspension containing the sample, lucigenin (5 µM), and assay phosphate buffer. Luminescence was measured in a plate luminometer (Auto-Lumat LB 953, Berthold Technologies, Bad Wildbad, Germany). The buffer blank was subtracted from each reading. Activity was expressed as relative light units per microgram of protein.

2.10 Quantitative real time PCR assay

The mRNA expression levels were determined by quantitative real-time PCR. Total RNA was obtained using TRIzol (Invitrogen Life Technologies). A total of 1 µg of DNase I-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 10-µl reaction. qRT-PCR was performed in duplicate for each sample using 0.5 µl cDNA as template, 1X iTaq™ Universal Probes Supermix (Biorad), and 20X Taqman Gene Expression Assays (COX-2: Rn00568225_m1, NOX-1: Rn00586652_m1, SOD-1: Rn00566938_m1, TXA₂ receptor: Rn00690601_m1, Applied Biosystems) in a 10-µl reaction using the following conditions: 2 min at 50°C and 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. qRT-PCR for eNOS (Fd: GAGAGTGAGCTGGTGTGG; Rv: GGTGAACATTCCTGTGCTGT) and NOX-2 (Fd: CCAGTGTGTCGGAATCTCCT; Rv: ATGTGCAATGGTGTGAATGG), were performed using the fluorescent dye SyBRGreen (iTaq FAST SyBRGreen Supermix with ROX, Bio-Rad, USA). For quantification, quantitative real-time PCR was carried out in a 7500 Fast (Applied Biosystems) adding a dissociation stage to the conditions already described above to show PCR product specificity. As a normalizing internal control, we amplified cyclophilin D (Rn01458749_g1). To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta C_t}$ method using untreated samples as a calibrator (Livak and

Schmittgen 2001). mRNA levels of the housekeeping gene were not modified by any of the treatments used.

2.11 Statistical analysis

Data are expressed as mean \pm SEM. In the vascular reactivity experiments, vasoconstrictor responses of aorta and MRA were expressed as a percentage of the contraction induced by 75 mM and 120 mM KCl, respectively. Vasodilator responses were expressed as a percentage of the previous contraction to Phe. To compare the effect of L-NAME, TEA, apocynin, SOD, indomethacin and NS-398 -on the response to Phe in segments from each group, some results were expressed as 'differences of area under the concentration-response curves' (dAUC) in control and experimental situations AUCs were calculated from the individual concentration response curve plots; differences were expressed as the percentage of the AUC of the corresponding control situation. Results were analyzed using unpaired Student's t-test or two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual means. Results of biochemical experiments were analyzed using Student's t-test or Mann-Whitney test. Values of $P < 0.05$ were considered significant.

3. Results

3.1 Body weight, fluid and feed intake and aluminum content

Body weight of rats was similar between groups at the beginning and end of treatments. Water or Al intakes as well as the feed intake were not different between groups (Martinez et al. 2017). The whole blood Al content was similar in rats treated with the low dose of Al (Untreated: 73.43 ± 1.7 vs Al 8.3: 77.1 ± 6.6 $\mu\text{g Al/L/blood}$, $P > 0.05$) while that of the rats treated with the high dose was greater than the corresponding control (Untreated: 78.9 ± 5.7 vs Al 100: 121.1 ± 11.2 $\mu\text{g Al/L/blood}$, $P < 0.05$). However, liver Al contents were greater both in the low (Untreated: 0.25 ± 0.01 vs Al 8.3: 0.44 ± 0.04 $\mu\text{g Al/g/dry tissue}$, $P < 0.05$) and also in the high Al-treated groups (Untreated: 0.26 ± 0.01 vs Al 100: 0.72 ± 0.01 $\mu\text{g Al/g/dry tissue}$, $P < 0.05$).

3.2 Aluminum raises systolic blood pressure

Exposure to Al at low (8.3 mg/kg bw for 60 days) or at high level (100 mg/kg bw for 42 days) increased SBP after the 3rd week of treatment (Untreated: 119.0 ± 1.4 vs Al 8.3: 129.2 ± 1.0 mmHg, $n=8$, $P < 0.05$; Untreated: 120.5 ± 1.5 vs Al 100: 131.5 ± 3.7 mmHg, $n=8$, $P < 0.05$). However, at the end of treatments only Al at the higher dose maintained this increase (Fig 1A, B).

3.3 Aluminum increases vascular reactivity and induces vascular dysfunction

Al exposure did not affect the response to KCl either in aorta (Untreated: 3.8 ± 0.3 vs Al 8.3: 4.1 ± 0.4 mN/mm; Untreated: 3.9 ± 0.2 vs Al 100: 4.1 ± 0.3 mN/mm; $n = 15$ to 20 , $P > 0.05$) or MRA (Untreated: 3.6 ± 0.2 vs Al 8.3: 3.5 ± 0.3 mN/mm; Untreated: 4.1 ± 0.3 vs Al 100: 4.5 ± 0.2 mN/mm, $n = 15$ to 20 , $P > 0.05$). However, Al treatment at both low and high levels increased the vasoconstrictor responses to Phe in aorta and MRA while decreased the endothelium-dependent responses induced by ACh only in MRA (Figs 2A, B, Table 1). The vasodilator response induced by SNP were unaffected by Al exposure (data not shown). These results suggest that Al affects vasoconstrictor responses in both conductance and resistance arteries and impairs endothelial function only in resistance arteries.

3.4 Aluminum decreases the endothelial modulation of the vasoconstrictor response – Involvement of endothelial NO synthase

To investigate whether Al exposure alters NO modulation on vasoconstrictor responses, the effect of endothelium removal and incubation with the NOS inhibitor L-NAME ($100 \mu\text{M}$), were investigated. Both endothelium removal and NOS inhibitor addition left-shifted the concentration–response curves to Phe in aorta and MRA segments from the four groups, but this effect was smaller in preparations from rats treated with either 8.3 mg/Kg or 100 mg/Kg of Al than in those from untreated rats, as shown by the dAUC values (Figs 3, 4). The endothelial modulation was impaired after Al exposure at an equivalent human dietary level and this effect was almost the same as observed after Al treatment at 12 times higher. Therefore, we decided to investigate the underlying mechanisms of Al toxicity by using the human relevant dose level and, Al exposure at 8.3 mg/kg decreased eNOS gene expression in aorta (Untreated: 0.85 ± 0.1 vs Al 8.3: 0.60 ± 0.1 relative expression, $P < 0.05$). To verify the influence of K^+ channels on the vasoconstrictor responses, aortic segments were incubated with TEA (2 mM), a K^+ channel blocker. The presence of TEA increased the contractile response to Phe in all groups, but this enhancement was smaller in aorta from Al-treated rats, as demonstrated by the dAUC values (Fig 5). All these findings suggest that the production and/or bioavailability of NO are impaired by Al exposure at both high and low level. The reduced participation of K^+ channels on vasoconstrictor responses follow the results of lower NO bioavailability, since NO can direct (Bolotina et al. 1994; Mistry & Garland 1998) or indirectly by cyclic GMP-dependent (Carrier et al. 1997) promote the activation of K^+ channel in MRA.

3.5 Aluminum increases oxidative stress – Role of NAD(P)H oxidase

The participation of oxidative stress in the vascular responses was evaluated *in vitro* using the non selective NAD(P)H oxidase inhibitor apocynin as well as SOD. Apocynin ($0.3 \mu\text{M}$) and SOD (150 U/ml)

reduced the vascular response to Phe in MRA from rats treated with both doses of Al, while those drugs did not affect the response in untreated animals (Fig 6); in aorta, both apocynin and SOD reduced the Phe-induced responses, being this effect greater in segments from Al-treated rats than from untreated rats, as demonstrated by the dAUC values (Fig 7). Al exposure either at low doses or at high doses increased vascular ROS and lipid peroxidation levels (ROS (UF): Aorta - Untreated: 98.4 ± 15.3 vs Al 8.3: 152.9 ± 9.7 ; Untreated: 98.4 ± 15.3 vs Al 100: 201.2 ± 24.1 ; MRA - Untreated: 129.2 ± 12.3 vs Al 8.3: 231.7 ± 22.7 , Untreated: 157.3 ± 13.2 vs Al 100: 228.5 ± 15.6 - n=8, $P < 0.05$; Lipid peroxidation (nM MDA/mg protein): Aorta - Untreated: 37.1 ± 2.1 vs Al 8.3: 50.3 ± 4.1 ; Untreated: 18.3 ± 0.6 vs Al 100: 30.6 ± 5.4 ; MRA - Untreated: 8.3 ± 1.3 vs Al 8.3: 15.7 ± 2.7 ; Untreated: 7.2 ± 1.0 vs Al 100: 20.3 ± 2.3 - n=8, $P < 0.05$). Al at both doses increased the MRA total antioxidant capacity (FRAP value (mM): Untreated: 32.1 ± 3.5 vs Al 8.3: 78.8 ± 4.3 ; Untreated: 35.9 ± 4.1 vs Al 100: 53.5 ± 5.9 - n= 8, $P < 0.05$), while in aorta the antioxidant capacity was reduced after Al exposure at 8.3 mg/kg (FRAP value (mM): Untreated: 64.1 ± 9.1 vs Al 8.3: 26.6 ± 4.0 $P < 0.05$, Untreated: 45.2 ± 2.5 vs Al 100: 42.9 ± 2.9 - n= 8, $P > 0.05$). Taking all together, these results suggest that the reduced NO bioavailability could be related with the increased oxidative stress after Al exposure.

NAD(P)H oxidase complex plays an important role on $O_2^{\bullet-}$ production (Martyn et al. 2006). Therefore, we investigated its possible role as source of ROS production after Al exposure. The basal $O_2^{\bullet-}$ production increased in both conductance and resistance arteries from rats treated with 8.3 mg/Kg (Fig 8). The NAD(P)H oxidase activity and the NOX1 gene expression were both increased in aorta from the Al-treated group compared to untreated group (Fig 9A,B); however, NOX2 mRNA was similar in treated and untreated animals (Fig 9C). Moreover, Al treatment inhibited the SOD1 gene expression in aorta from Al-treated animals (Fig 9D).

3.6 Aluminum vascular impairment - Participation of COX-2-derived prostanoids

To investigate the role of prostanoids on the increased response to Phe in Al-treated rats, the cyclooxygenase inhibitor indomethacin and the selective COX-2 inhibitor NS 398 were used. Indomethacin (1 μ M) and NS 398 (1 μ M) reduced the vasoconstrictor response to Phe in both arteries from all groups. However, as observed by the dAUC values, this reduction was greater in segments from both Al-treated groups when compared to the untreated groups (Fig 10, 11), suggesting an increased participation of contractile prostanoids from COX-2 on Phe-induced responses. Al exposure at the human relevant level of 8.3 mg/kg increased the mRNA levels of both COX-2 and TXA-2 R in aorta (Fig 12). These results suggest

that COX-2-derived prostanoids, probably acting on TP receptors, play a role on the vascular impairment observed after Al exposure at human dietary relevant level.

4. Discussion

The present study provides evidence that Al should be considered as a risk to the cardiovascular system even at low doses. Here, we show that Al exposure for 60 days at human dietary levels increased blood pressure and vascular reactivity in both conductance and resistance vessels through endothelium-dependent mechanisms. Moreover, these effects were almost the same when we treated rats with Al at a dose 12 times higher (100 mg/kg), representing a high level of human exposure to Al. We demonstrate that ROS and COX-2-derived prostanoids are important mediators of aorta and mesenteric vascular dysfunction observed after Al exposure. The excess of ROS mainly from NAD(P)H oxidase and the increased vascular COX-2 act in concert to decrease NO bioavailability, thus inducing vascular dysfunction and increased blood pressure.

Due to the omnipresence of Al in our daily life, human exposure to Al is still underestimated (Exley 2013). The tolerable weekly intake of Al for humans has been set at 1 mg Al/Kg bw (EFSA 2008; WHO 2007), which may be easily exceeded by humans due to myriad sources of Al exposure (Exley 2013; Fekete et al. 2013; Gonzalez-Weller et al. 2010; Yang et al. 2014). We have recently established an experimental model of exposure to Al that mimics human exposure to this metal through diet in which rats were exposed to Al at a dose of 8.3 mg/Kg bw *per day* during 60 days. Considering the amount of Al present in the animal's feed, the total Al exposure was 4.37 mg/Al/day (2.49 mg from water plus 1.88 mg from feed). In addition, we have compared this low dose with a high level of human exposure to Al (100 mg/Kg bw), in which rats were treated with a total of 31.88 mg/Al/day (30 mg from gavage plus 1.88 mg from feed) (Martinez et al. 2017). In the present study, Al exposure at low dose did not change the whole blood Al content between groups; however it promoted an increased Al accumulation in the liver. The lack of differences for blood contents after Al exposure at a low dose is expected since blood is only a temporal storage organ for Al (Beardmore and Exley 2009). The high deposition of Al in the liver even without differences in the blood level supports these findings. Of interest, the human blood Al content is extremely variable in the available literature, ranging from less than 1 to 901 µg Al/L of blood (Chen et al. 2013; Lind et al. 2012).

Cardiovascular risk and human body burden of Al share the characteristic that both rise with increase of average lifetime (Assmann et al. 2017; Exley 2013). Therefore, considering the postulated mechanisms

of action of Al and the underlying ones regarding vascular disease, for example increased oxidative stress, finding more relations between them would not be a surprise. In previous years, studies have analyzed the possible cardiovascular adverse effects of Al exposure. Isolated rat hearts exposure to Al at 100 μ M showed a reduction of both coronary blood flow and isovolumetric systolic pressure (Gomes et al. 1994). The effects of Al on heart function was further analyzed and the perfusion of hearts with Al at 40 μ M caused an increase in coronary flow and both reduction in heart rate and increases in pulsatile power (Korchazhkina et al. 1998).

More recently, Al was related to the development of hypertension in experimental animals, these effects were related to an increase in renin expression or erythrocyte membrane impairments after acute or chronic Al administration up to 256 mg/kg (Ezomo et al. 2009; Zhang et al. 2015). In humans, this possible relationship was also seen, where circulating plasma Al levels were related to arterial hypertension (Granadillo et al. 1995). In a recent study, our group showed that one hour of Al exposure at 100 mg/kg is sufficient to promote vascular changes with reduction in vascular reactivity and increase in vascular ROS production (Schmidt et al. 2016). To our knowledge, our study is the first experimental one investigating and showing cardiovascular effects after Al exposure at human relevant dietary levels. The current study supports the possible cardiovascular risk of Al. Thus, the main results show that Al exposure: 1) increases systolic blood pressure; 2) produced endothelial dysfunction, as shown by the impaired the vasodilator response to ACh and the increased vasoconstrictor responses to Phe and 3) reduced the endothelial NO bioavailability.

Al³⁺ toxicity has been related with its pro-oxidant activity in different target organs and systems in experimental animals (Prakash and Kumar 2009; Yu et al. 2016). The pro-oxidant effects of Al are well documented and are possible due to formation of superoxide radical ion (Exley 2004) or by promoting the Fenton reaction by reducing Fe(III) to Fe(II) (Ruipérez et al. 2012). It is well known that oxidative stress alters vascular reactivity through several ways such as its effects on the NO pathway, by counteracting NO effects or by reducing its bioavailability (Hernanz et al. 2014). NO can rapidly react with O₂^{•-}, promoting the formation of the powerful oxidant ONOO⁻, leading to the loss of the vasodilator effects of NO (Alvarez et al. 2008; Zou 2007). In the present study, the suggested reduced NO bioavailability could be due to the inhibited eNOS gene expression or due to a direct effect of the increased oxidative stress.

NADPH oxidases play a central role in the production of vascular superoxide radical anion and hydrogen peroxidase (Konior et al. 2014). The isoforms NOX-1 and NOX-2 expressed in mammals seem

to trigger the development of vascular pathologies such as atherosclerosis, hypertension, neurological disorders, inflammation and cancer (Konior et al. 2014; Schramm et al. 2012). In our study, the *in vitro* exposure to apocynin and to the antioxidant SOD promoted a higher reduction in the vasoconstrictor response to Phe in segments from AI-treated rats, suggesting the involvement of the superoxide radical anion, presumably from NADPH oxidase, on vascular dysfunction induced by AI. This was further support by the fact that the basal $O_2^{\cdot-}$ production, the mRNA levels of NOX-1 subunit and the NADPH oxidase activity were increased while the mRNA levels of cytosolic Cu/Zn SOD was decreased in AI-treated rats. Moreover, the vascular redox imbalance suggested by the increased ROS and lipid peroxidation as well as by the antioxidant capacity alterations, support that AI induced pro-oxidant mechanisms likely explained the observed vascular alterations.

Vascular tone is critically modulated by COX-derived prostanoids and its vascular effects in physiological and pathological conditions are depending on the activation of specific receptors (Hernanz et al. 2014; Avendaño et al. 2016). Recently, a circuitous relationship between COX-2 products and ROS acting to induce vascular dysfunction in hypertension was shown (Martinez-Revelles et al. 2013). The toxicity effects of AI have been strongly related with its pro-oxidant capacity (Exley 2004; Ruipérez et al. 2012; Yu et al. 2016) and, recently an involvement of COX-2 pathway in the neuro-pathological effect of AI has been suggested (Yu et al. 2014; Wang et al. 2015). In the present study we found that the respective unspecific and specific inhibitors of COX-2, indomethacin and NS 398, promoted a reduction in the vasoconstrictor response to Phe mainly in aortic and MRA segments of AI-treated rats. These results suggests the participation of COX-2 derived prostanoids in the vascular dysfunction observed after AI exposure. In agreement, aortas from AI-treated rats showed an increase on mRNA levels of COX-2 and TXA-2 R. The TXA-2 R is involved in critical regulations of the vascular wall such as platelet aggregation and smooth muscle contraction and vascular changes of hypertension (Félétou et al. 2011; Nakahata 2008). Here, its activation seems to play a role in vascular alterations found in AI-treated rats, however further experiments are necessary to better address this hypothesis.

5. Conclusions

Our study provides evidence that 60-day exposures to low doses of AI, which aimed to mimic human exposure to AI by dietary source, are able to compromise cardiovascular health. The current study shows that AI increases systolic blood pressure and vascular reactivity through endothelium-dependent mechanisms. Here, we demonstrate that ROS and COX-2-derived prostanoids are important mediators of

vascular dysfunction after Al exposure. The excess of ROS mainly from NAD(P)H oxidase and increased vascular COX-2 seem to participate in the vascular alterations after Al exposure. Both act in concert to decrease NO bioavailability, which in turn induces vascular dysfunction and increases blood pressure. Therefore, our findings provide a better understanding of the cardiovascular risk of human exposure to Al.

Conflict of Interest: The authors declare that they have no conflict of interest.

Acknowledgments

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Figure legends

Figure 1. Effect of chronic aluminum exposure on non-invasive systolic blood pressure. Values of systolic blood pressure (mmHg) of untreated rats and treated with AlCl_3 for 60 (8.3 mg/kg bw *per day* - A) or 42 days (100 mg/kg bw *per day* - B). Data are expressed as mean \pm SEM, $n=8$, * $P < 0.05$ compared with their corresponding controls (untreated rats) (Two-Way ANOVA followed by Bonferroni).

Figure 2. Effect of chronic aluminum exposure on vascular reactivity. Concentration–response curves to (A) phenylephrine and (B) acetylcholine in aorta and MRA segments. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ compared with their corresponding Untreated groups (Two-Way ANOVA followed by Bonferroni).

Figure 3. Effect of chronic aluminum exposure on NO-mediated vascular response in MRA. Effects of (A) endothelium removal (E–) and (B) L-NAME (100 μM) on the concentration-response curve to phenylephrine from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) endothelium-denuded and intact segments and (B) in the presence and absence of L-NAME. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ vs control

curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ vs Untreated (Student's t-test) in dAUC graphs.

Figure 4. Effect of chronic aluminum exposure on NO-mediated vascular response in aorta. Effects of (A) endothelium removal (E-) and (B) L-NAME (100 μ M) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration-response curves (dAUC) in (A) endothelium-denuded and intact segments and (B) in the presence and absence of L-NAME. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ vs control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ vs Untreated (Student's t-test) in dAUC graphs.

Figure 5. Effect of chronic aluminum exposure on K⁺ channels action on the vascular response in aorta. Effects of (A) the K⁺ channels blocker TEA (2 mM) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration-response curves (dAUC) in (A) in the presence and absence of TEA. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ vs control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ vs Untreated (Student's t-test) in dAUC graphs.

Figure 6. Effect of chronic aluminum exposure on ROS-mediated vascular response in MRA. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μ M) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine in MRA from untreated and Al-treated rats. The inset shows differences in the area under the concentration-response curves (dAUC) in (A) in the presence and absence of apocynin and (B) in the presence and absence of SOD. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ vs control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ vs Untreated (Student's t-test) in dAUC graphs.

Figure 7. Effect of chronic aluminum exposure on ROS-mediated vascular response in aorta. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μ M) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration-response curves (dAUC) in (A) in the presence and absence of apocynin and (B) in the presence and absence of SOD. Data are expressed as mean \pm SEM, $n=8$ to 15, * $P < 0.05$ vs control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ vs Untreated (Student's t-test) in dAUC graphs.

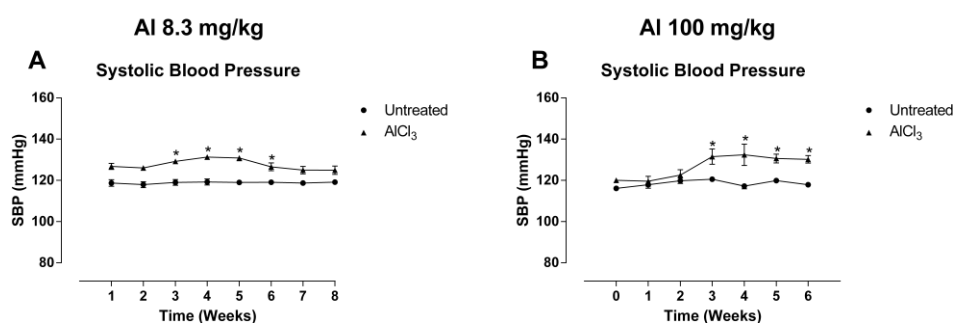
Figure 8. Effect of chronic aluminum exposure on vascular O₂⁻ production. Representative fluorescent photomicrographs of arterial sections labeled with the oxidative dye hydroethidine and vascular superoxide anion quantification in MRA (A) and aorta (B) from untreated and Al-treated rats (8.3 mg/kg – 60 days). Data are expressed as mean \pm SEM ($n = 8$). * $P < 0.05$ compared with the untreated group (Student's t-test). Au: Fluorescence intensity.

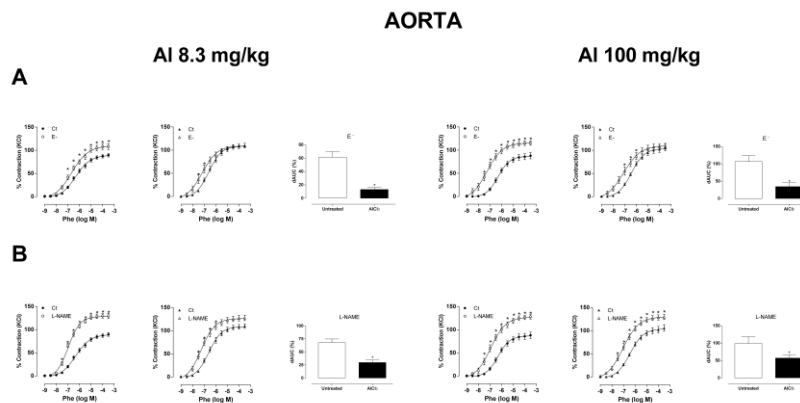
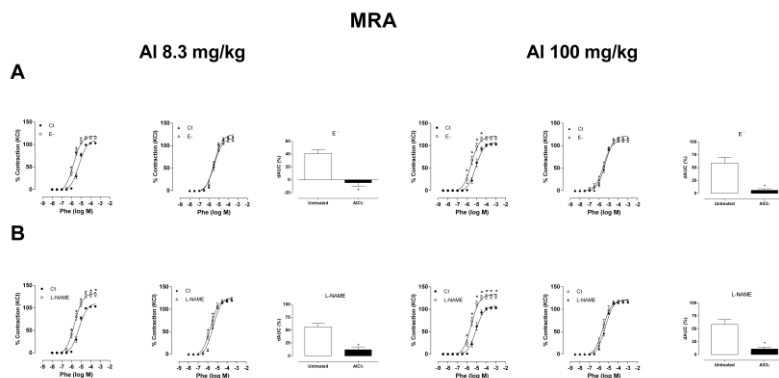
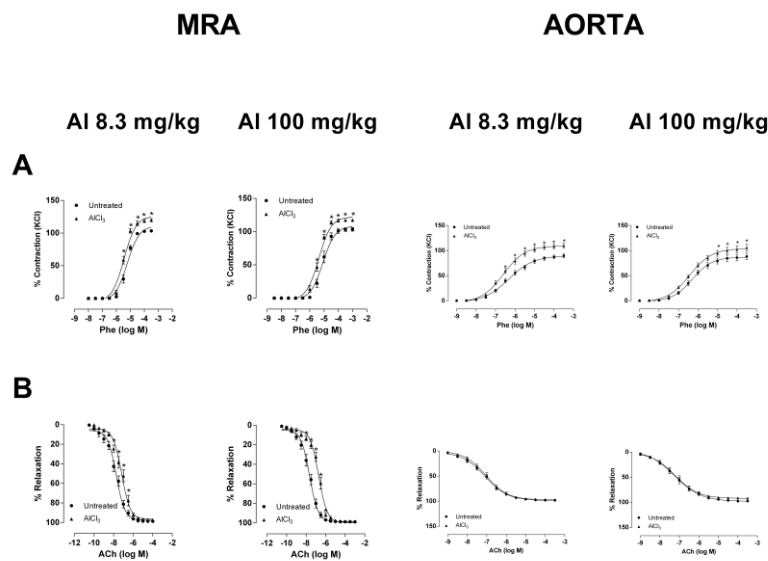
Figure 9. Effect of chronic aluminum exposure on NOX oxidase enzyme activity, NOX-1, NOX-2 and SOD 1 gene expressions. NOX oxidase activity (A) and mRNA levels for NOX-1 (B), NOX-2 (C) and SOD 1 (D) in aortas from untreated and Al-treated rats (8.3 mg/kg – 60 days). Data are expressed as mean \pm SEM (n = 8). * P < 0.05 compared with the untreated group (Student's t-test).

Figure 10. Effect of chronic aluminum exposure on COX-derived prostanoids role on vascular response in MRA. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μ M) and (B) selective COX-2 inhibitor NS 398 (1 μ M) on the concentration– response curve to phenylephrine in MRA from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of indomethacin and (B) in the presence and absence of NS 398. Data are expressed as mean \pm SEM, n=8 to 15, * P < 0.05 vs control curve (Two-Way ANOVA followed by Bonferroni) * P < 0.05 vs Untreated (Student's t-test) in dAUC graphs.

Figure 11. Effect of chronic aluminum exposure on COX-derived prostanoids role on vascular response in aorta. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μ M) and (B) selective COX-2 inhibitor NS 398 (1 μ M) on the concentration– response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of indomethacin and (B) in the presence and absence of NS 398. Data are expressed as mean \pm SEM, n=8 to 15, * P < 0.05 vs control curve (Two-Way ANOVA followed by Bonferroni) * P < 0.05 vs Untreated (Student's t-test) in dAUC graphs.

Figure 12. Effect of chronic aluminum exposure on COX-2 and TXA-2 R gene expressions. COX-2 mRNA levels (A) and TXA-2 R mRNA levels (B) in aortas from untreated and Al-treated rats (8.3 mg/kg – 60 days). Data are expressed as mean \pm SEM (n = 8). * P < 0.05 compared with the untreated group (Student's t-test).



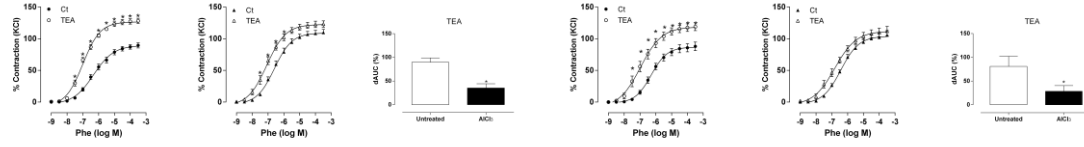


AORTA

AI 8.3 mg/kg

AI 100 mg/kg

A

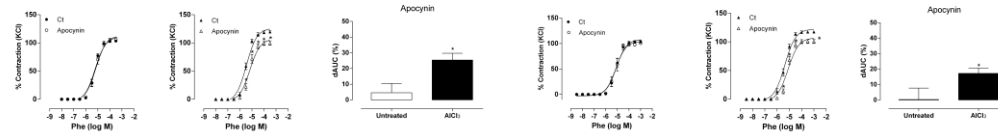


MRA

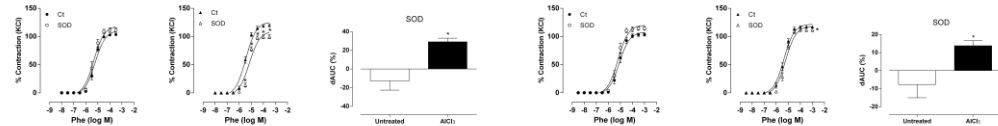
AI 8.3 mg/kg

AI 100 mg/kg

A



B

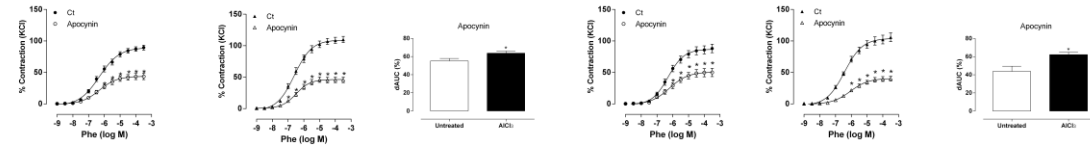


AORTA

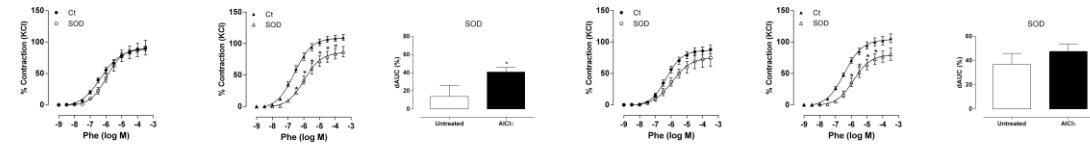
AI 8.3 mg/kg

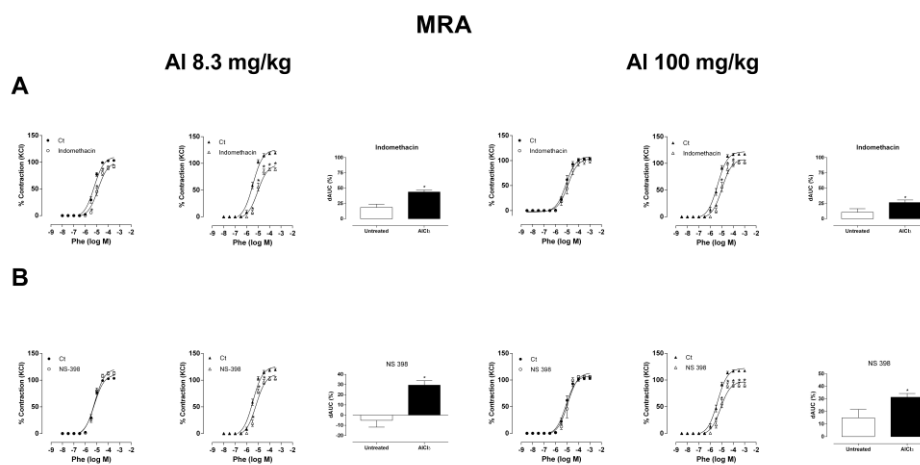
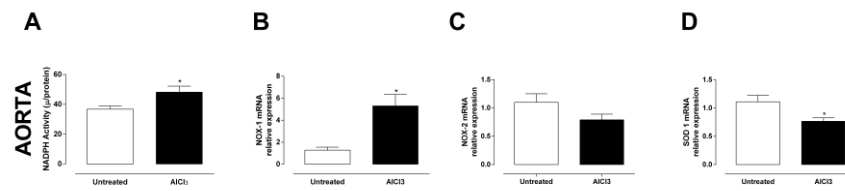
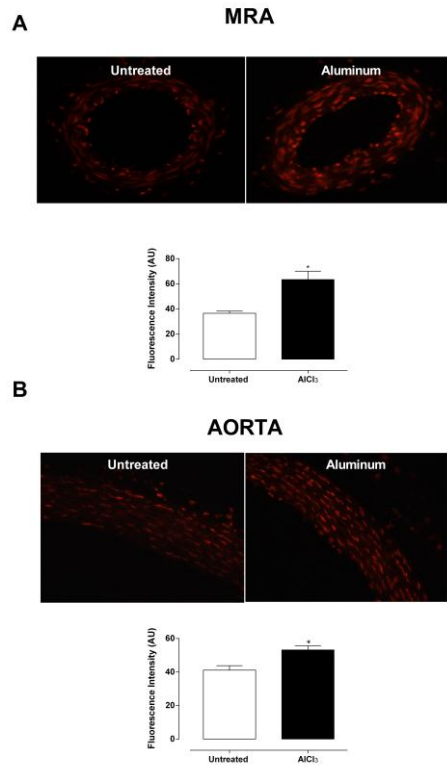
AI 100 mg/kg

A



B





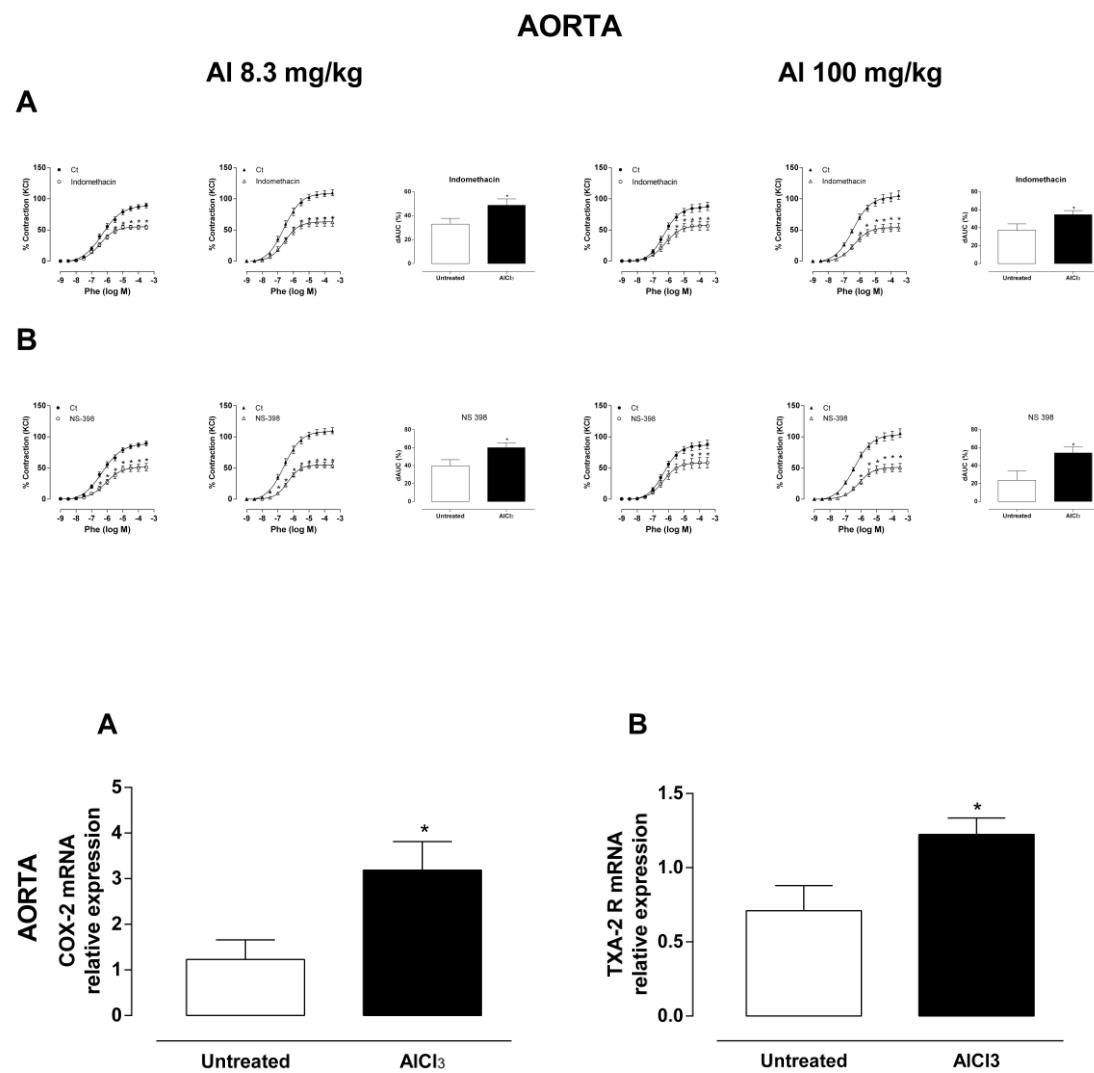


Table 1 Effects of aluminum exposure to low and high doses on maximum response (R_{max}) and sensitivity (pD_2)

	Untreated		Al 8.3 mg/kg		Untreated		Al 100 mg/kg	
	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2
Aorta								
Control	89.7 ± 4.3	6.3 ± 0.1	111.1 ± 5.1 [#]	6.5 ± 0.1	86.7 ± 6.5	6.3 ± 0.1	107.2 ± 7.4 [#]	6.4 ± 0.1
E-	110.6 ± 6.2*	6.7 ± 0.1*	109.1 ± 2.4	7.1 ± 0.1*	116.6 ± 5.5*	7.1 ± 0.1*	108.5 ± 6.1	7.1 ± 0.2*
L-NAME	129.5 ± 5.1*	6.9 ± 0.1*	126 ± 5.5	7.2 ± 0.1*	127.3 ± 4.6*	7.1 ± 0.2*	129.2 ± 5.5*	7.1 ± 0.1*
TEA	126.9 ± 4.2*	7.0 ± 0.0*	123.5 ± 5.1	7.1 ± 0.1*	118.1 ± 5.8*	7.2 ± 0.3*	112.1 ± 7.1	7.0 ± 0.2*
Apocynin	44.6 ± 5.4*	6.3 ± 0.1	46.6 ± 4.2*	6.4 ± 0.1	50.3 ± 6.3*	6.2 ± 0.1	41.3 ± 4.0*	5.9 ± 0.1*
SOD	89.2 ± 11.2	6.0 ± 0.1	89.7 ± 8.3*	5.8 ± 0.1*	74.9 ± 13.3	6.0 ± 0.1	81.6 ± 9.8	5.7 ± 0.1*

Indomethacin	55.1 ± 3.3*	6.4 ± 0.1	62.9 ± 6.6*	6.5 ± 0.1	56.9 ± 7.3*	6.1 ± 0.1	54.3 ± 6.4*	6.2 ± 0.18
NS 398	51.3 ± 5.9*	6.1 ± 0.1	55.1 ± 4.6*	6.3 ± 0.1	58.2 ± 8.3*	6.3 ± 0.1	50.8 ± 6.5*	6.1 ± 0.1*
MRA								
Control	111 ± 1.8	4.2 ± 0.2	123 ± 3.4 [#]	5.1 ± 0.2 [#]	109.1 ± 3.1	3.7 ± 0.2	120 ± 2.7 [#]	4.7 ± 0.3 [#]
E-	120 ± 3*	5.1 ± 0.4	117.1 ± 2.8	4.1 ± 0.3	123.3 ± 4.5*	5.4 ± 0.4*	114.4 ± 2.8	4.3 ± 0.3
L-NAME	130.4 ± 4.7*	5.3 ± 0.4*	126.9 ± 5.1	5.1 ± 0.4	135.2 ± 6.2*	5.1 ± 0.6	122.4 ± 3.6	5.1 ± 0.3
Apocynin	116.2 ± 3.9	3.9 ± 0.3	114.3 ± 3.4*	4.3 ± 0.1*	119.5 ± 4.2	4.9 ± 0.2*	115.5 ± 3.2	4.4 ± 0.3
SOD	129.1 ± 3.5*	5.2 ± 0.3*	125 ± 4.6	4.9 ± 0.5	135 ± 5.8	5.3 ± 0.1*	123.5 ± 3.6	4.8 ± 0.2
Indomethacin	103.3 ± 3.1*	3.1 ± 0.3*	105.3 ± 3.1*	2.9 ± 0.3*	111.1 ± 7.6	4.1 ± 0.5	114.8 ± 5.2	4.1 ± 0.3
NS 398	125.2 ± 2.7*	4.7 ± 0.2	118.4 ± 3.3*	4.4 ± 0.2	129.7 ± 0.3*	4.7 ± 0.3	128.8 ± 2.4	5.2 ± 0.7

Parameters of maximal response (R_{\max}) and sensitivity (pD_2) of the concentration-response curves to phenylephrine in both aorta and MRA before (control) and after endothelial damage (E-), L-NAME (100 μ M), TEA (2 mM), apocynin (0.3 μ M), SOD (150 U/ml), Indomethacin (1 μ M) and NS 398 (1 μ M) incubations. Results are expressed as mean \pm SEM. R_{\max} : maximal effect (expressed as a percentage of maximal response induced by KCl) and pD_2 expressed as a -log one-half R_{\max} . * $P < 0.05$ compared to the corresponding control in each group # $P < 0.05$ compared with the Untreated group (Student's t-test).